

**REMARKS**

After entry of the Preliminary Amendment, claims 8-20 are pending in this application.

**I. Amendments to the Specification**

The amendment on page 1 complies with Applicants' obligations under 35 U.S.C. § 120 to claim priority in the first paragraph of the specification.

The amendments to the specification on pages 6, 12, 18, 26 and 27 are typographical in nature, are made to place the application in better form for allowance, and do not add prohibited new matter.

The amendment to page 23 clarifies the nomenclature used in the table. Support for this change is presented in the specification on page 10, lines 20 and 25 and on page 11, line 4, and would be commonly understood by the skilled artisan. No prohibited new matter is added.

**II. Amendments to the Claims**

The amendments to the claims merely redraft the claims in independent form (claims 8-16) or correct the referenced dependency to the renumbered claims.

In addition, the word "gene" has been replaced by the word "DNA (claims 8-14), support for which is found at page 17, lines 12-16 and page 17, lines 26 to page 18, line 10.

The acronyms "BFP" or "GFP" have been replaced by the term "fluorescent," as was done for the allowed claims (U.S. Patent 6,194,548) to eliminate redundant terminology and is supported by the disclosure at page 1, lines 6-7 and page 6, lines 1-2.

No prohibited new matter is added by these amendments or claims.

**III. Claim for Priority**

Under the provisions of Section 119 of 35 U.S.C., Applicants hereby claim the benefit of the filing date of Japanese Patent Application No. 10-026418, filed January 23, 1998.

In support of Applicants' claim for priority, Applicants rely on the certified copy of Japanese Patent Application No. 10-026416 filed in United States Patent Application Serial No. 09/121,539, parent application to the instant divisional application.

**CONCLUSION**

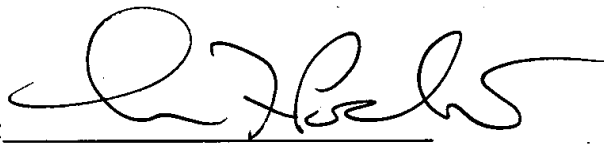
For the reasons set forth above, entry of the foregoing amendment and examination of the application respectfully is requested. If there are any other filing or claim fees due in connection with the filing of this preliminary amendment, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for any extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

The Examiner is invited to telephone either of the undersigned if a discussion might expedite placing the claims in condition for allowance.

Respectfully submitted,

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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Specification:**

Paragraph at page 6, lines 18-24 has been amended as follows:

In view of the above-mentioned problems, the present inventors [~~made extensive researches~~] performed extensive research and succeeded in the discovery of novel GFPs and BFPs that are free from such problems by introducing certain mutations into specific positions of the amino acid sequence for GFP or BFP, thus accomplishing this invention.

Heading at page 12, line 16 has been amended as follows:

**DESCRIPTION OF THE ~~[PREFRED]~~ PREFERRED EMBODIMENTS**

Paragraph at page 18, lines 13-21 has been amended as follows:

Moreover, methods for randomly introducing mutations are not particularly limited, and Mutagenic PCR as described below can preferably be used in this invention. The Mutagenic PCR can be carried out according to methods known in the art. (C.W. Dieffenback, ed. PCR PRIMER, A Laboratory Manual (Cold Spring Harbor Laboratory Press) [~~(1955)~~] (1995) pp. 583-588.) Concretely, the following conditions were employed in the examples.

Paragraph at Page 18, line 22 through Page 19, line 8 has been amended as follows:

About 50 ng of Plasmid BlueBFP (201) was added to [~~10xmutagenic~~] 10 x mutagenic PCR buffer (70 mM MgCl<sub>2</sub>, 500 mM KCl, and 100 mM Tris-HCl, pH 8.3 at 25 °C; 0.1% (w/v) gelatin) 10 µl, [~~10x dNTP~~] 10 x dNTP (2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM dTTP) 10 µl, 10 mpol/µl primer (23mer M13Universal primer and M13Reverse primer) 3µl, and H<sub>2</sub>O 62µl, and mixed. Subsequently, 10 µl of 5mM MnCl<sub>2</sub> was added and mixed, and 1µl of Taq Polymerase (Takara) was added to conduct PCR (PC-700 available from ASTEC Inc. was used). The PCR was conducted in three tubes under the following conditions: 25 cycles at 94 °C for 1 min, 30 cycles at 45 °C for 1 min, and 35 cycles at 72 °C for 1 min, respectively.

The Table 4 on Page 23, lines 5-20 has been amended as follows:

TABLE 4

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GFP						
101	none					
103		Phe64Leu				
104			Val163Ala		Ser175Gly	
105		Phe64Leu,		Val163Ala,	Ser175Gly	
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BFP (as for BFP, the two mutations, [ <del>Y66H</del> ] <u>Tyr66His (Y66H)</u> and [ <del>Y145F</del> ] <u>Tyr145Phe (Y145F)</u> , have been introduced into the sequence for GFP which serves as a basis)						
201	Y66H,	Y145F:				
202	Y66H,	Y145F:	Phe64Leu,	Leu236Arg		
203	Y66H,	Y145F:	Phe64Leu			
204	Y66H,	Y145F:		Val163Ala,	Ser175Gly	
205	Y66H,	Y145F:	Phe64Leu,	Val163Ala,	Ser175Gly,	Leu236Arg
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Paragraph at Page 25, line 19 through Page 26, line 19 has been amended as follows:

Unless otherwise so stated, CHO-K1 cells were grown in a F12+10% FBS medium in 5% CO<sub>2</sub> at 37 °C. The cells [~~(1×10<sup>5</sup>)~~](1 × 10<sup>5</sup>) were inoculated into a 6-cm dish, and on the following day, their transfection was conducted in two dishes as a pair by the calcium phosphate method. (C. Chen and H. Okayama Mol. Cell. Biol. 7: 2745-2752 (1987).) After transfection, the one dish was incubated at 37 °C and the other at 30 °C for 24 h. The transfected CHO cells were washed with [~~1×PBS (-)~~] 1 × PBS (-) three times, and they were dissolved in 1 ml of 10 [~~mM Tris-HCl~~] mM Tris-HCl (pH 7.4) containing 1% Triton X-100 and recovered in an Eppendorf tube. A supernatant (0.5 ml) from centrifugation at 3,000 rpm for 5 min was diluted 4-fold with the same buffer and fluorescence measurement was performed. Here, a pUcD2SRαMCS vector (empty vector) was transfected and used as a blank. A Hitachi F-2000 type fluorophotometer was used in the fluorescence measurement. In the measurement of GFPs, fluorescence was scanned between 460 nm and 600 nm at an excitation wavelength of 460 nm to

measure the maximal value in the vicinity of the fluorescence wavelength of 510 nm. In the measurement of BFPs, fluorescence was scanned between 360 nm and 500 nm at an excitation wavelength of 360 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 445 n.

Paragraph at Page 26, line 21 through Page 27, line 22 has been amended as follows:

The CHO cells were transfected with pUcD2SRαMCS (empty vector)(T. Tsukamoto et al. Nature. Genet. 11: 395-401 (1995)), phGFP(101)-Cl, phGFP(105)-Cl, phBFP(201)-CL, and phBFP(205)-Cl, respectively and grown at 37 °C and at 30 °C. Employing a sample prior to dilution as used in the fluorescence measurement previously described (8 μl), SDS-PAGE was performed on a 12% gel. With the use of a Horizonblot (ATTO Inc.), transfer was conducted onto a nitrocellulose membrane (Millipore Inc., HAHY394FO) under the conditions of 2 mA and 90 min per cm<sup>2</sup>. After the membrane was taken out and washed with [~~1x~~PBS] 1 × PBS, it was immersed in 1% skim milk/PBS and shaken at room temperature for 30 min. After the membrane was washed with [~~1x~~PBS] 1 × PBS, it was immersed in 0.1% skim milk/PBS containing an anti-GFP antibody (Clonotech Inc.) that had been diluted 2,000-fold and shaken at 4 °C overnight. The membrane was washed with [~~1x~~PBS] 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was immersed in 0.1% skim milk/PBS containing an anti-rabbit IgG antibody labeled with HRP (Amersham Inc.) that had been diluted 1,000-fold, and shaken at 4 °C for 1 h. The membrane was washed with [~~1x~~PBS] 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was reacted with a chemiluminescence reagent (Amersham Inc. ECL) for 1 min, and then, was exposed to an X-ray film for 2 min.

**In the Claims:**

Claim 8 has been amended as follows:

8. (Amended) [~~A-gene~~] DNA encoding [~~the-GFP~~] a fluorescent protein [~~according to claim 1~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Phe64Leu, Val163Ala, and Ser175Gly.

Claim 9 has been amended as follows:

9. (Amended) [~~A-gene~~] DNA encoding [~~the-GFP~~] a fluorescent protein [~~according to claim 2~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with the three mutations of Phe64Leu, Val163Ala, and Ser175Gly.

Claim 10 has been amended as follows:

10. (Amended) [~~A-gene~~] DNA encoding [~~the-BFP~~] a fluorescent protein [~~according to claim-3~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, and Phe64Leu.

Claim 11 has been amended as follows:

11. (Amended) [~~A-gene~~] DNA encoding [~~the-BFP~~] a fluorescent protein [~~according to claim-4~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

Claim 12 has been amended as follows:

12. (Amended) [~~A-gene~~] DNA encoding [~~the-BFP~~] a fluorescent protein [~~according to claim-5~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with the four mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

Claim 13 has been amended as follows:

13. (Amended) [~~A-gene~~] DNA encoding [~~the-BFP~~] a fluorescent protein [~~according to claim-6~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.

Claim 14 has been amended as follows:

14. (Amended) [~~A-gene~~] DNA encoding [~~the-BFP~~] a fluorescent protein [~~according to claim-7~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with the six mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.